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FOREWORD

First and foremost, we would like to extend our sincere appreciation and utmost gratitude to Associate Professor Dr. Ngah Ramzi Hamzah, Rector of UiTM (Pulau Pinang), Dr. Mohd Mahadzir Mohammud@Mahmood, Deputy Rector of Academic Affairs and Dr. Mohd Subri Tahir, Deputy Rector of Research, Industry, Community & Alumni Network for their generous support towards the successful publication of this issue. Not to be forgotten also are the constructive and invaluable comments given by the eminent panels of external reviewers and language editors who have worked assiduously towards ensuring that all the articles published in this issue are of the highest quality. In addition, we would like to thank the authors who have submitted articles to EAJ, trusting Editor and Editorial Board and thus endorsing a new initiative and an innovative academic organ and, in doing so, encouraging many more authors to submit their manuscripts as well, knowing that they and their work will be in good hands and that their findings will be published on a short-term basis. Last but not least, a special acknowledgement is dedicated to those members of the Editorial Board who have contributed to the making of this issue and whose work has increased the quality of articles even more. Although there will always be cases in which manuscripts will be rejected, our work so far has shown that the board members' motivation has been, and will be, to make publications possible rather than to block them. By means of intensive communication with authors, academic quality is and will be guaranteed and promising research findings are and will be conveyed to the academia in a functional manner.

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PROTECTIVE EFFECT OF *STEVIA REBAUDIANA* LEAF EXTRACT ON HEMOLYTIC BEHAVIOUR OF HUMAN ERYTHROCYTE IN HYDROGEN PEROXIDE-INDUCED OXIDATIVE STRESS

Norhisham Haron[1], Nuraishah Abu Hasan[2], Siti Nazrina Camalxaman[3], Emida Mohamed[4], Azlin Sham Rambely[5] and Zulkhairi Amom[6]

[1, 2, 3, 4, 5]Department of Medical Laboratory Technology, Faculty of Health Sciences, Universiti Teknologi MARA, 42300 Bandar Puncak Alam, Selangor, Malaysia.
[6]Department of Basic Science, Faculty of Health Sciences, Universiti Teknologi MARA, 42300 Bandar Puncak Alam, Selangor, Malaysia.
[1]hishamharon@puncakalam.uitm.edu.my; [2]nuraishahabuhasan@gmail.com:
[3]sitinazrina@salam.uitm.edu.my; [4]mohamedemida@yahoo.com;
[5]azlinshamrambely@yahoo.com; [6]zulkha2992@puncakalam.uitm.edu.my

ABSTRACT

Stevia rebaudiana, a member of Asteraceae family is rich in stevioside and total phenolic compounds. The aim of the present study was to investigate the protective effect of Stevia rebaudiana leaf extract on human erythrocytes against oxidative damage, in vitro. The protective effect of Stevia rebaudiana leaf extract in erythrocytes was studied in terms of resistance to hemolysis, lipid peroxidation and morphological changes. Stevia rebaudiana leaf extract at different concentrations (100, 200 and 300 µg/mL) was tested in 10 mM hydrogen peroxide (H_2O_2) induced oxidative stress on human erythrocytes. The hemolysis of erythrocytes induced by H_2O_2 was inhibited in a dose-dependent manner. The Stevia rebaudiana leaf extract was found to inhibit erythrocyte's lipid peroxidation caused by H_2O_2. Morphological alterations in erythrocytes induced by H_2O_2 were protected by the leaf extract. In conclusion, the study demonstrated that Stevia rebaudiana leaf extract protected erythrocytes against oxidative damage due to its antioxidant properties.

Keywords: *Stevia rebaudiana* extract; antioxidant; human erythrocytes; oxidative stress; hemolysis.

1. INTRODUCTION

Oxidative stress can be described as an imbalance between the production of reactive oxygen and the biological system's ability to repair the damage (Bains & Shaw, 1997). It has been reported that oxidative damage may play an important pathophysiological role in many types of human diseases (Winkler, Boulton, Gottsch, & Sternberg, 1999). An increase in oxidative stress may lead to irreversible damage of erythrocytes and could result in their hemolysis and removal from circulation (Gradinski-Vrbanac et al., 2002). The reactions between reactive

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oxygen species and lipids in cellular membranes cause lipid peroxidation which may contribute to the loss of elasticity and increased membrane osmotic fragility (Jain, 1984).

Erythrocytes have been used as a cellular model to investigate oxidative damage in biomembrane. Due to the presence of high polyunsaturated fatty acid content in membranes and high cellular concentrations of oxygen in hemoglobin, erythrocytes are considered extremely susceptible to oxidative damage (Battistelli et al., 2009). Lipid peroxidation caused by reactions of reactive oxygen species on lipids membrane can be monitored by the levels of malondialdehyde (MDA). This highly reactive product is shown to cause a cross linking of membrane proteins which may impair the function of erythrocytes membrane and lead to reduce erythrocytes survival (Singh & Rajini, 2008). The exposure of free hemoglobin to H_2O_2 will cause heme degradation with the release of iron ions that initiate free radical and lipid peroxidation that induce abnormalities in erythrocyte shape (Ajila & Prasada Rao, 2008).

On the other hand, several plants have been reported to contain phenolic compounds which are capable to scavenge free radicals and inhibit the process of lipid peroxidation (Rice-Evans, Miller, & Paganga, 1997). According to Osawa (1994) the antioxidant activities of phenolic compounds are due to their redox properties which are able to absorb and neutralize free radicals, quench singlet and triplet oxygen or decompose peroxide. Similarly, flavonoids and other phenolic compounds have the ability to protect the bilayer membrane from damage induced by detergent and free radical mediated lipid oxidation (Erlejman, Verstraeten, Fraga, & Oteiza, 2004; Oteiza, Erlejman, Verstraeten, Keen, & Fraga, 2005; Erlejman, Fraga, & Oteiza, 2006).

Stevia rebaudiana, a member of Asteraceae family, is a sweet herb native to the valley in highlands of Paraguay and Brazil but has been cultivated in East Asia including in China, Korea, Thailand and Malaysia due to its beneficial health properties (Kujur et al., 2010). Extract from the leaf has been reported to contain flavonoids, alkaloids, water-soluble chlorophylls and xanthophylls, hydroxycynnamic acids, free sugars, neutral-water soluble oligosaccharides, lipids, amino acids, essential oils and trace elements (Komissarenko, Derkach, Kovalyov, & Bublik, 1994). It also displays a potent antihypertensive (Chan et al., 2000; Lee et al., 2001), antihyperglycemic (Jeppesen, Gregersen, Alstrupp, & Hermansen, 2002), antimicrobial (Satishkumar, Saravanan, & Seethalakshmi, 2008) and antioxidant activities (Kim, Yang, Lee, & Kang, 2011). Due to the fact that *Stevia rebaudiana* contains high total phenolic compounds and possesses antioxidative properties, this study aims to determine the protective effect of stevia leaf extract against free radical mediated-H_2O_2 induced oxidative damage in normal human erythrocytes.

2. MATERIALS AND METHODS

2.1 Materials

Stevia rebaudiana leaves were obtained from Agro Sweet Pak Long Sdn. Bhd. Kajang, Malaysia after being authenticated by plant taxonomist. OxiSelect™ TBARS Assay Kit for MDA quantitation was purchased from Cell Biolabs Inc., USA. Hydrogen peroxide was purchased from Sigma Aldrich Co., USA. All other chemicals used were of analytical grade.

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2.2 Preparation of Stevia Extract

10 g of air-dried powder of *Stevia rebaudiana* leaf was soaked in 100 mL of ethanol and incubated at room temperature for 24 h (Shukla, Mehta, & Bajpai, 2009). The mixture was filtered using Whatman no. 1 filter paper and concentrated to dryness by rotary evaporator at 45 ℃. The extract was then dissolved in dimethyl sulphoxide (DMSO) to make a stock solution. Three different concentrations of *Stevia rebaudiana* leaf extract (100, 200 and 300 µg/mL) were freshly prepared from the stock by mixing the stock solution with 10mM PBS (pH 7.4).

2.3 Preparation of Erythrocytes

Erythrocytes from healthy consenting donors were collected in heparinized tubes and immediately centrifuged at 1500 *g* for 10 min at 4 ℃. Following this, the plasma and buffy coat were removed, the erythrocytes were washed in 10 volumes of 10mM PBS (pH 7.4) followed by centrifugation at 1500 *g* for 5 min at 4 ℃. This process was repeated three times. Supernatant and buffy coats of white cells were carefully removed within each wash. The final product of washed erythrocytes were stored at 4 ℃ and used within 6 h for subsequent analyses (Ajila & Prasada Rao, 2008). All the experiments were carried in accordance to the ethical clearance approval from Universiti Teknologi MARA ethical committee.

2.4 Evaluation of Hemolysis

The protective role against erythrocytes hemolysis by the *Stevia rebaudiana* leaf extract was evaluated according to the procedure described by Ajila and Prasada Rao (2008). 50 µL of each extract (100, 200 and 300 µg/mL in 10 mM PBS pH 7.4) was added to 100 µL of 10% (v/v) suspension of erythrocytes. 100 µL of 10 mM H_2O_2 (in 10 mM PBS pH 7.4) was then added. The mixture was incubated for 3 h in a shaker at 37 ^{0}C. The erythrocytes were treated with 10 mM H_2O_2 and without extract to obtain a complete hemolysis. The erythrocytes incubated with PBS served as control. After incubation, the mixture was diluted with 8 mL PBS and then centrifuged at 2000 *g* for 10 min. Erythrocytes hemolysis was evaluated by measuring the haemoglobin (Hb) concentration in supernatants at 540 nm spectrophotometrically. Percentage of hemolysis was calculated considering hemolysis caused by 10 mM H_2O_2 as 100%.

2.5 Membrane Lipid Peroxidation

Membrane lipid peroxidation was evaluated by the production of thiobarbituric acid reactive substances (TBARS). The TBARS level was determined by the equivalent malondialdehyde (MDA) standards, according to the recommendations of test manufacturer OxiSelect™ TBARS Assay Kit (MDA Quantitation). Briefly, 100 µL of the unknown MDA containing samples or MDA standards were allowed to react with thiobarbituric acid (TBA) at 95 ℃ for 1 h. Following incubation, the samples and standards were cooled and supernatant was read spectrophotometrically at 532 nm. The MDA concentration in the samples was extrapolated from the predetermined MDA standard curve.

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2.6 Erythrocytes Morphology

50 µL of the packed erythrocytes were incubated in a tube containing 100 µL of 10mM H_2O_2 with or without extract (50 µL) for 60 min at 37 ℃. After incubation, the mixtures were centrifuged at 2000 *g* for 10 min and the pellets were processed for blood smear. The smear was air dried followed by staining using the Leishman method (Dacie & Lewis, 1984). The slides were viewed under LEICA light microscope and images of the erythrocytes were digitally recorded and analyzed. The pathological shape of the erythrocytes such as echinocyte and stomatocytes were identified and calculated based on the number of abnormal cells per total number of erythrocytes.

2.7 Statistical Analysis

Results are presented as mean ± standard error mean (SEM) from three independent experiments. One way ANOVA, followed by Scheffe's test was used to determine the difference of means. $P < 0.05$ was considered to be statistically significant.

3. RESULTS

3.1 Stevia Rebaudiana Protection against Hemolysis

The protective effect of *Stevia rebaudiana* leaf extract on H_2O_2 induced erythrocytes hemolysis is shown in Figure 1.The exposure of cells to 10 mM H_2O_2 for 3 h caused complete cellular hemolysis, whereas minimum hemolysis was observed (19 ± 0.3 %) in the control group. Meanwhile, groups treated with stevia extract at all concentrations showed a significant reduction of erythrocyte hemolysis (50 ± 2.1 %, 39 ± 0.6 % and 32 ± 1.0 %) respectively compared to the H_2O_2-induced group ($P<0.05$). The reduction of hemolysis was found to be in a dose-dependent manner.

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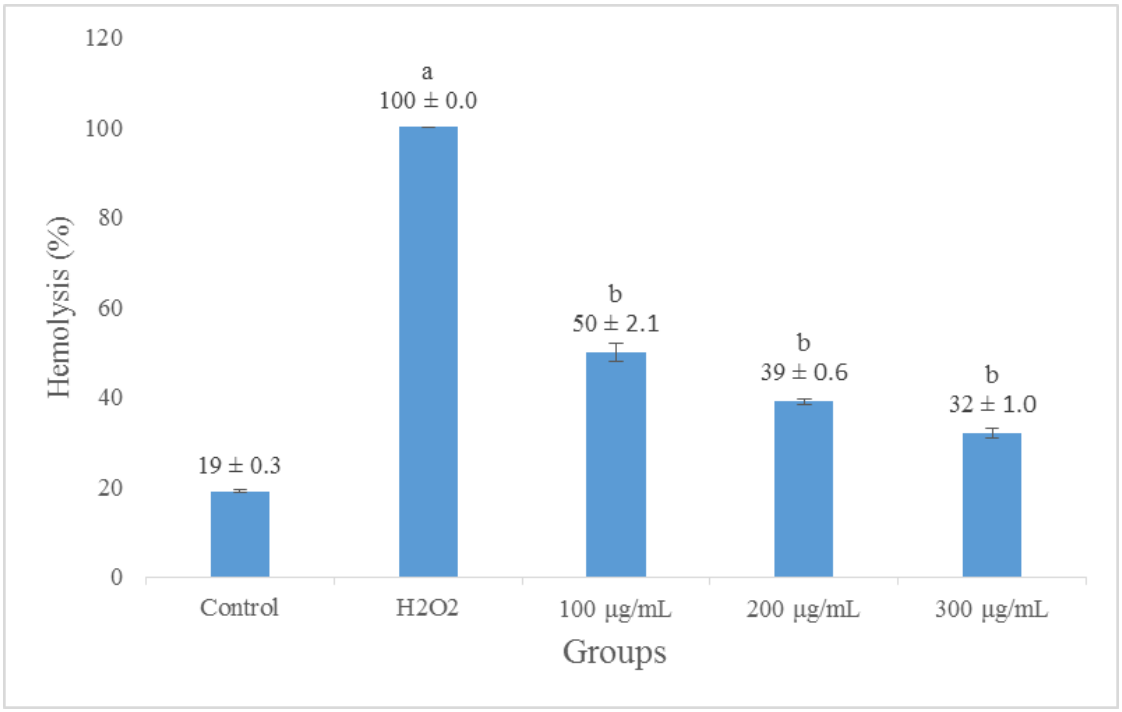

Figure 1: Protective effect of *Stevia rebaudiana* leaf extract on the hemolysis of human erythrocytes induced by 10mM H_2O_2. Percentage hemolysis are reported as mean ± S.E.M (n = 3). $^{a}p < 0.05$ compare to control, $^{b}p < 0.05$ compare to H_2O_2.

3.2 Stevia Rebaudiana Protection against Lipid Peroxidation

Lipid peroxidation in erythrocyte was determined by the quantification of malondialdehyde (MDA) molecules, a natural by-product of lipid peroxidation. As shown in Figure 2, there is a significant elevated of MDA concentration in the supernatant of group exposed to H_2O_2 compared to the control group ($P<0.05$). On the other hand, treatment of mixture with stevia extract illustrates a significantly low MDA concentration compared to the induced group ($P<0.05$) and the reduction was found to be in a dose-dependent manner. The MDA concentration in H_2O_2-induced groups treated with *Stevia rebaudiana* leaf extract (100, 200 and 300 µg/mL) were 5.18 ± 0.03 µM, 4.22 ± 0.04 µM and 1.88 ± 0.06 µM respectively.

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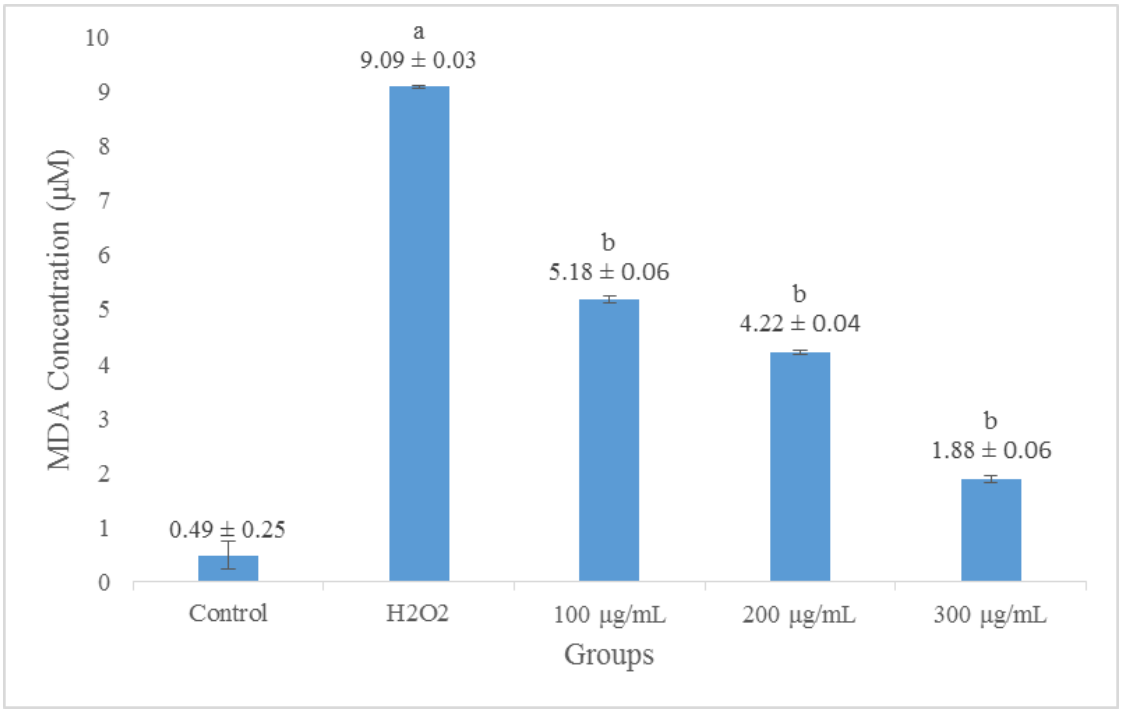

Figure 2: Protective effect of *Stevia rebaudiana* leaf extract on the lipid peroxidation of human erythrocytes induced by 10mM H_2O_2. Results are expressed as mean ± S.E.M (n = 3). $^{a}p < 0.05$ compare to control, $^{b}p < 0.05$ compare to H_2O_2.

3.3 Stevia Rebaudiana Protection against Morphological Changes

The morphological structure of erythrocytes was observed under light microscope to evaluate the cellular alterations in different experimental conditions. The percentage of abnormal morphology and images of erythrocytes treated with H_2O_2 and *Stevia rebaudiana* leaf extract are shown in Figure 3 and Figure 4. The control group, which was not exposed to 10 mM H_2O_2 demonstrated a typical erythrocyte characterized with normal biconcave discoid (discocyte) shape. On the other hand, the cells that were exposed to 10 mM H_2O_2 resulted in significant alteration in the shape, mainly as echinocyte and stomatocyte form (46 ± 3.8% of abnormal cells) compared to the control group ($P<0.05$). The groups treated with stevia extract at 200 and 300 µg/mL showed a significant reduction of altered erythrocytes compared to the induced group ($P<0.05$). Schistocyte, cells with fragmented and irregularly shaped seen with intravascular hemolysis, were also present in the sample incubated with 100 µg/mL of the extract. In fact, the percentage of abnormal cells in group treated with 300 µg/mL of *Stevia rebaudiana* leaf extract and in the control group was slightly similar.

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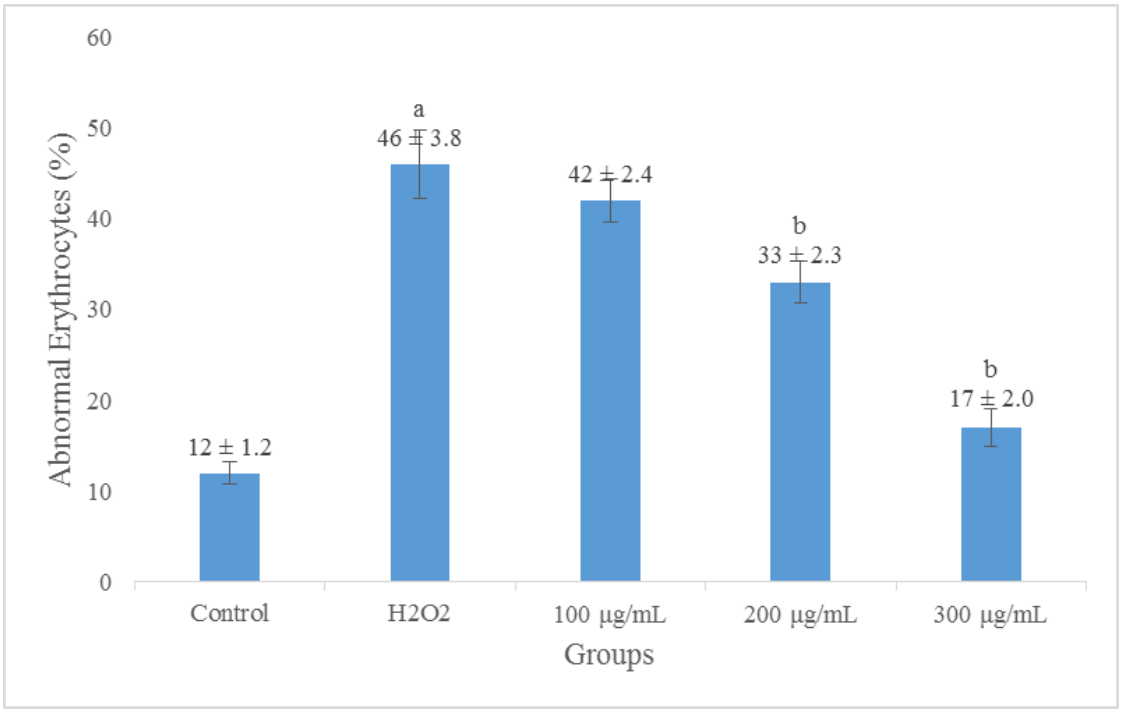

Figure 3: Protective effect of *Stevia rebaudiana* leaf extract on the morphological changes of human erythrocytes induced by 10mM H_2O_2. The protection percentage are expressed as mean ± S.E.M (n = 3). $^{a}p < 0.05$ compare to control, $^{b}p < 0.05$ compare to H_2O_2.

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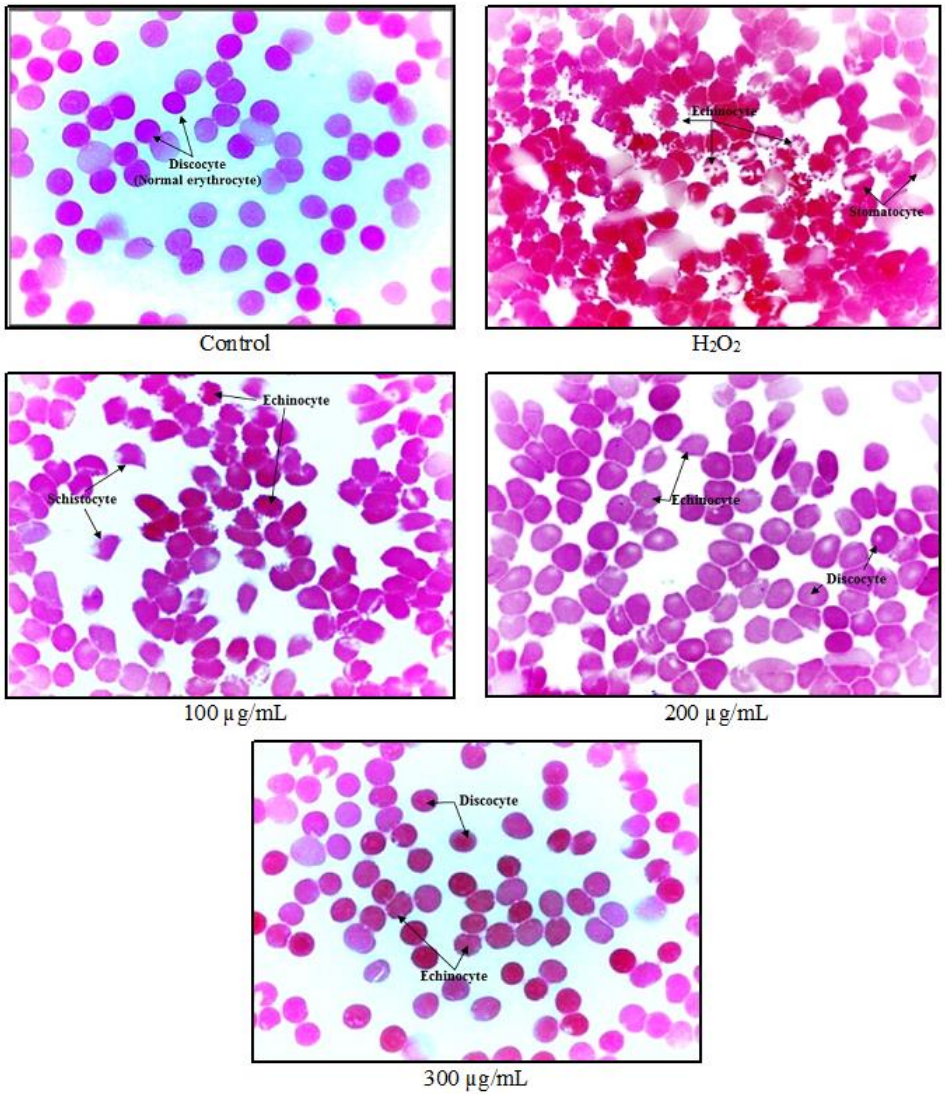

Figure 4: Light microscope images of normal human erythrocytes and protective effects of different concentrations of *Stevia rebaudiana* leaf extract against H_2O_2 induced oxidative damage on erythrocytes (x100).

4. DISCUSSION

Erythrocytes are vulnerable to reactive oxygen species due to the presence of high polyunsaturated fatty acid content in their membranes. Oxidation of erythrocytes by reactive oxygen species lead to lipid peroxidation, changes in cellular morphology, protein cross-linking, hemolysis and conformation of membrane proteins (Okamoto et al., 2004). Phenolic compounds in plants have been reported to provide a cellular defence system against oxidative injury (Alvarez-Suarez et al., 2012; S. M. Nabavi, S. F. Nabavi, Eslami, & Moghaddam, 2012). In the present study, erythrocytes were chosen as the *in vitro* models to study the protective effects of the *Stevia rebaudiana* leaf extract as inhibitor of oxidative damage induced by H_2O_2.

This experiment illustrated the toxicity effect of 10 mM H_2O_2 and induces cellular lysis in normal human erythrocytes. A significant increase in the percentage of hemolysis was observed in H_2O_2-induced group further proves that ferryl radical or hydroxyl radical is

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responsible to cause cellular damage such as alterations in cell shape, membrane deformability, deterioration of phospholipid organization and cell surface characteristics (Synder et al., 1985). Van den Berg, Lubin, Roelofsen and Kuypers (1992) demonstrated that interactions of radicals with hemoglobin resulted in erythrocytes lysis. Furthermore, erythrocytes with an increased metHgb concentration, lipid peroxidation and spectrin-Hgb complexes can also be seen (Cimen, 2008). The study shows that the co-incubation of the mixture with *Stevia rebaudiana* leaf extract has caused a significant decrease in the percentage of cellular hemolysis. The beneficial effects of the extract are possibly due to the antioxidant compounds that are present in the extract that are able to scavenge free radicals. In fact, high concentrations of total phenolic compounds in stevia leaf acts as a reducing agent that is crucial for quenching and terminating the radical chain reactions (Tadhani, Patel, & Subhash, 2007). Study by Zeng, Cai, Yang and Wu (2013) reported that the ethanolic extracts of *Stevia rebaudiana* leaf have natural antioxidants that include phenolics, flavonoids and other substances. Besides, the hydrophobicity of the extract is important to protect the hydrophobic part of the cell membrane like lipid-soluble chain-breaking antioxidants. This may principally scavenge radicals in the lipophilic regions of erythrocytes membranes, thus protecting them from lysis (Blasa, Candiracci, Accorsi, Piacentini, & Piatti, 2007). Hence, the alcoholic extract of stevia leaf might possibly exhibit their antioxidant effects according to the degree of liposolubility.

According to Baynes (2005), oxidants are reported to initiate lipid peroxidation reactions in cells which can lead to loss of membrane integrity and cell death. The thiobarbituric acid reactive substances (TBARS), a by-product of lipid peroxidation can be detected by the TBARS assay using thiobarbituric acid as a reagent. The assay measures malondialdehyde (MDA) generated from lipid hydroperoxides by the hydrolytic conditions of the reaction. MDA is a well-known product of lipid peroxidation that is commonly used as a marker of oxidative stress. In this study, the increase in the-concentrations of MDA in erythrocytes upon H_2O_2 treatment is in accordance with the data reported by Ajila and Prasada Rao (2008). In addition, the free radical generated from H_2O_2 reacts with membrane lipids, leads to the initiation of lipid peroxidation processes, while the cell membranes with high polyunsaturated fatty acids are susceptible to oxidizing radicals (Cheeseman & Slater, 1993). Consequently, the lipid hydroperoxide which is formed from the lipid peroxidation can cause detrimental effect such as a cross linking of membrane proteins and a change in membrane fluidity (Beckman & Ames, 1997). In the present study, the reduced MDA concentration in H_2O_2-induced erythrocytes treated with stevia extracts suggests that the antioxidant potential from *Stevia rebaudiana* leaf has the ability in neutralising the free radicals, chelating catalytic metals and scavenging free radical electrons and superoxides (Buyukokuroglu, Oktay, & Kufrevioglu, 2001; Thomas & Glade, 2010). According to Marar (2011) the solubility of ethanolic extract of *Stevia rebaudiana* leaf in lipophilic environment and its ability to penetrate to a specific site in the erythrocytes membrane has further extended the antioxidant activity by stevia against highly reactive radicals in the hydrophobic phase. The oxidative damage on erythrocytes causes abnormalities in erythrocyte shape and rheological properties by lowering the cytoskeletal protein content and production of high molecular weight proteins (Battistelli et al., 2009). In this study, a significant difference was observed in the percentage of abnormal shape of the cells when treated with H_2O_2. The observation showed that echinocyte and stomatocyte are the major abnormal cells in H_2O_2-induced oxidative stress. According to the bilayer couple hypothesis (Sheetz & Singer, 1974; Lim, Wortis, & Mukhopadhyay, 2002), the changes of erythrocytes shape when induced by foreign molecules

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are due to the differential expansion of the two monolayer of the erythrocyte membrane. Hence, echinocytes are formed when the oxidant is inserted into the outer leaflet of the erythrocytes membrane, whereas stomatocytes are produced when it is located into the inner monolayer of the membrane. Earlier finding has reported that, the spectrin-hemoglobin complex is formed following the peroxidation of membrane protein by H_2O_2. The condensation of the inner monolayer lipids by the spectrin-hemoglobin complex leads to a decrease in the inner lipid monolayer area which results in echinocyte formation (Singh & Rajini, 2008). On the other hand, the morphological alterations of erythrocytes induced by H_2O_2 are significantly prevented when treated with *Stevia rebaudiana* leaf extract. It is observed that the protective effect of stevia on cellular morphological changes is driven in a dependent manner. In fact, the co-incubation experiment may offer a protective effect of stevia extract both in the intracellular and extracellular environment (Battistelli et al., 2009). Duthie and Crozier (2000) stated that, since the primary mechanism of a phenolic antioxidant is to trap and stabilize free radicals species, the protective effect of the extract may also be related to localization of the phenolic compounds within the cell membrane of the erythrocytes. The interaction of extract components with membrane may alter the rigidity of the membrane which will result in membrane stability (Arora, Byrem, Nair, & Strasburg, 2000; Youdim, Shukitt-Hale, MacKinnon, Kalt, & Joseph, 2000). Furthermore, the restriction of membrane fluidity by the phenolic compounds may hinder the diffusion of H_2O_2. Thus, it will decrease the kinetics of free radical reactions (Suwalsky, Orellana, Avello, & Villena, 2007). A considerable antioxidant activity of the ethanolic extract of stevia leaf could be attributed to their capacity to scavenge free radicals in the erythrocytes membrane.

5. CONCLUSION

The present study, demonstrates that *Stevia rebaudiana* leaf extract protects the erythrocytes hemolysis against H_2O_2 induced oxidative stress. The extract also shows protection against lipid peroxidation and morphological changes of erythrocytes caused by H_2O_2. The *in vitro* protection of erythrocytes against oxidative damage suggests that stevia extract might be a valuable natural antioxidant in the prevention and treatment of various conditions related to oxidative stress. Thus, studies on the compounds responsible for the antioxidant activity and the mechanisms by which they protect against disease development are highly warranted.

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